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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The epidermal growth factor receptor (EGFR) plays an important role in receptor transactivation events initiated by G protein-coupled receptors (GPCRs), integrins and cytokine receptors. In these signaling pathway, the EGFR frequently is responsible for activating Ras and MAP kinase/extracellular signal-regulated kinase (ERK). EGFR transactivation may be ligand-dependent, in which case EGF or TGFα bind to the receptor, cause its dimerization and activation, or ligand-in-dependent, due to the activity of intracellular kinases, such as c-Src, which induce tyrosine phos-phorylation of the EGFR. EGFR and c-Src are cooverexpressed in a wide range of human tumors including the brain, lung, breast and prostate. Much evidence suggests that overexpression of TGFα and its cognitive receptor EGFR is involved in the later stages of human breast cancer and may play a role in growth and metastatic processes. Results from recent studies using cultured fibroblasts and human breast cancer cell lines have indicated that c-Src and EGFR synergistically interact to promote tumor formation in nude mice xenografts. To check whether this synergism occurs in the more physiological setting of the mammary gland, I am testing the interaction of these tyrosine kinases in transgenic mouse models, where MMTV EGFR, c-Src and TGFα transgenic mice generated. MMTV EGFR transgenic mice will be cross-bred with MMTV-c-Src and/or TGFα transgenic mice to form bigenic or trigenic mice, then examined for tumor formation in the mammary gland tissue. If the synergism hypothesis is correct, bigenic mice should develop large tumors more rapidly than single transgenic mice, and trigenic more rapidly then bigenic mice thus validating the synergism between c-Src, EGFR and TGFα as targets for future therapies.

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Introduction

Malignant transformation and progression of human cancer is frequently associated with overabundance of proteins that are involved in normal cellular processes, such as proliferation, differentiation, migration or apoptosis. Examples of such proteins include members of human epidermal growth factor receptor (hEGFR) and cellular Src (c-Src) tyrosine kinase families, which are frequently cooverexpressed in human neoplasms and especially in breast cancer. Much evidence suggests that overexpression of TGFα and its cognitive receptor EGFR is involved in the later stages of human breast cancer and may play a role in growth and metastatic processes. Results from recent studies using cultured fibroblasts and human breast cancer cell lines have indicated that c-Src and EGFR synergistically interact to promote tumor formation in nude mice xenografts. To check whether this synergism occurs in the more physiological setting of the mammary gland, I am testing the interaction of these tyrosine kinases in transgenic mouse models, where MMTV EGFR, c-Src and TGFα transgenic mice will be generated. MMTV EGFR transgenic mice will be cross-bred with MMTV-c-Src and/or TGFα transgenic mice to form bigenic or trigenic mice, then examined for tumor formation in the mammary gland tissue. If the synergism hypothesis is correct, bigenic mice should develop large tumors more rapidly than single transgenic mice, and trigenic more rapidly then bigenic mice, thus validating the synergism between c-Src, EGFR and TGFα as targets for future therapies.

Ī. Research accomplishments associated with the tasks outlined in the approved Statement of work.

Task 1. To construct MMTV-c-Src and MMTV-EGFR transgenes and test their expression in cell culture.
Task 1 was completed last year as it was reported previously (see annual fellowship report 2004)

Task 2. To generate and maintain MMTV-c-Src and MMTV-EGFR transgenic mice.

a) As a have reported previously, I have generated MMTV-EGFR transgenic mice. Now I have a stable colony for 2 years with 100% transmission of the transgene.

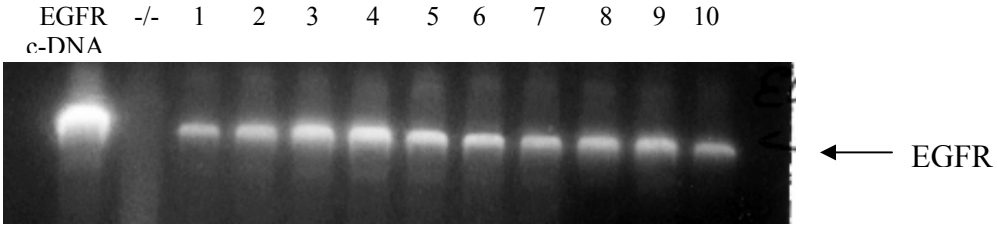


Figura A. Transgene integration into mouse genomic DNA

Transgene integration into mouse genomic DNA was analyzed by PCR using human EGFR specific primers as described before (see previous report) 1-10 samples of transgenic mice genomic DNA, EGFR- plasmid DNA, positive control; -/- non-transgenic mice genomic DNA, negative control

b) MMTV c-Src transgenic mice, derived from MMTV-pMSG-Src plasmid, did not yield any positive pups. None of 3 additional microinjections were successful either. PCR and Southern hybridization analysis suggested that c-Src did not integrate into the germ line and the animals were mosaic. It is also possible that the c-Src transgene could be toxic to developing mice.
To overcome this problem, I made a new c-Src plasmid construction, using a different (BSL1- STOP VENUS) vector, obtained from T. Bender of UVA. BSL1- STOPVENUS-c-Src will be expressed in all tissues of mice, and detection of expression will be facilitated by the fluorescent VENUS protein, which is an intensely fluorescent variant of GFP. VENUS theoretically can be expressed in tissues, but it will be used as tracking for c-Src expression. Breeding of BSL1- STOPVENUS-c-Src mice with MMTV-Cre transgenic mice will result in

targeted expression of c-Src (and VENUS) in hormone responsive tissues, such as mammary glands. Cre recombinase will excise the STOP signal located between two lox P sites upstream of c-Src gene, allowing transcription from the EF1 promoter.

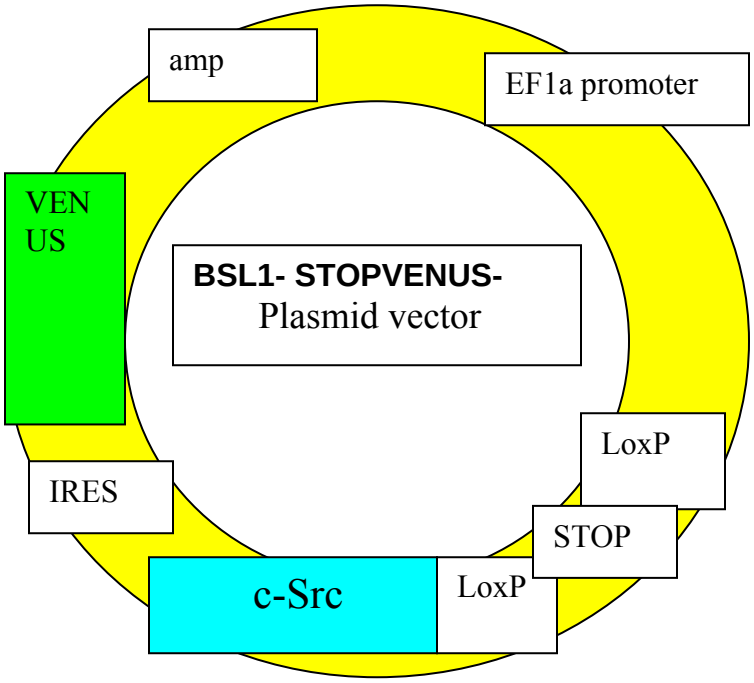
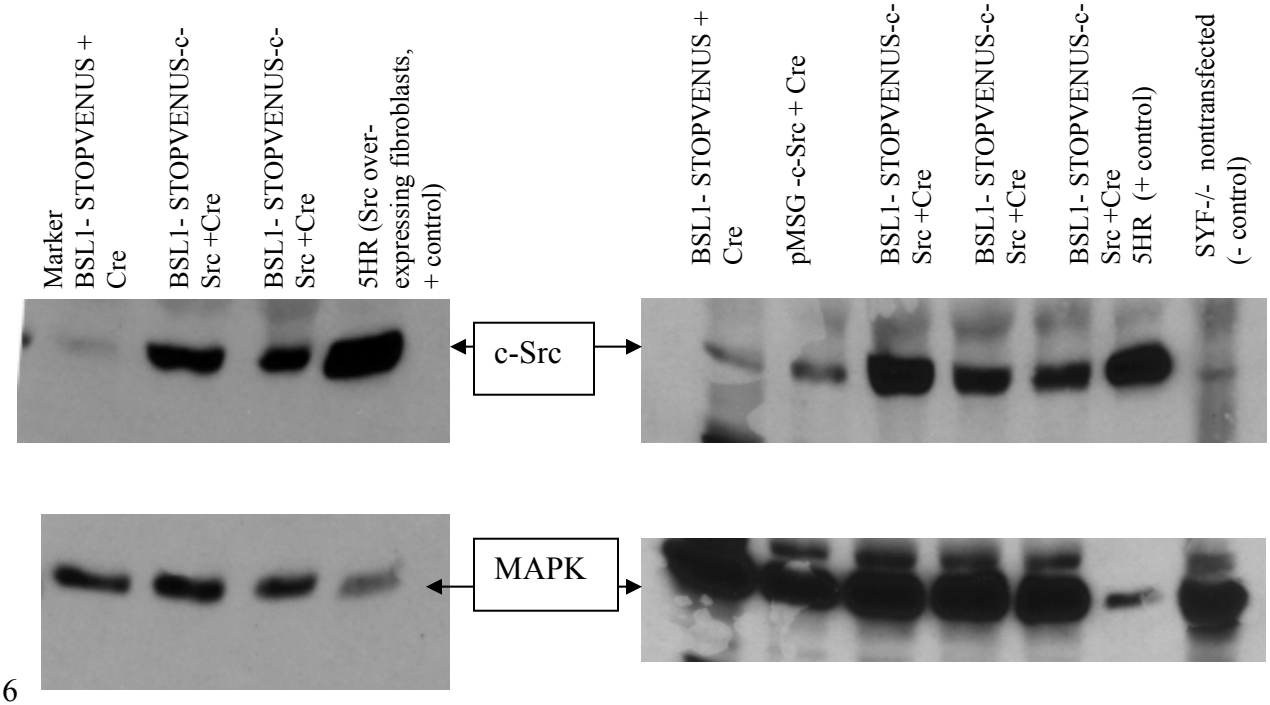


Figure B. BSL1- STOPVENUS-c-Src plasmid vector.

Then I tested the Cre inducible expression of this construction by transient co-transfection of BSL1- STOPVENUS-c-Src and Cre plasmid into COS-7 cells and SYF -/- (Src, Yes, Fyn -/-) fibroblasts and Western Blotting for c-Src overexpression and MAPKinase as a loading control (Figure C)



COS-7 cells SYF -/- fibroblasts

Figure C. BSL1- STOPVENUS-c-Src plasmid was appropriately expressed in both cell types in presence of Cre recombinase

After that PCR reaction was performed in order to detect the presence of c-Src in the BSL1- STOPVENUS-c-Src cDNA against a background of total mouse genomic DNA with the sensitivity of one copy of cDNA/genome.

As a control for the detection of the presence of a chicken c-Src cDNA stably integrated into mouse genomic DNA, PCR was performed using chicken c-Src primers and genomic DNA from mouse fibroblast 10T1/2 cell lines that have an integrated copy of chicken c-Src cDNA.

chicken c-Src primers

5' – ACCCCCAACAAGACAGCAGCC – 3'
5' – CAAAGTCAGAAACGGAGAGGC –3'

Chicken-specific primers for c-Src were used for PCR reactions to detect the presence of the transgene, against a mouse background. The c-Src primers gave a PCR product of about 0.587 kb and encompassed base pairs 99 through 686. I used genomic DNA from 2 cell lines as templates – 10T1/2 and 5H mouse fibroblasts. 10T1/2 – are parental mouse fibroblasts expressing endogenous mouse c-Src; 5H are 10T1/2 cells stably expressing chicken c-Src.

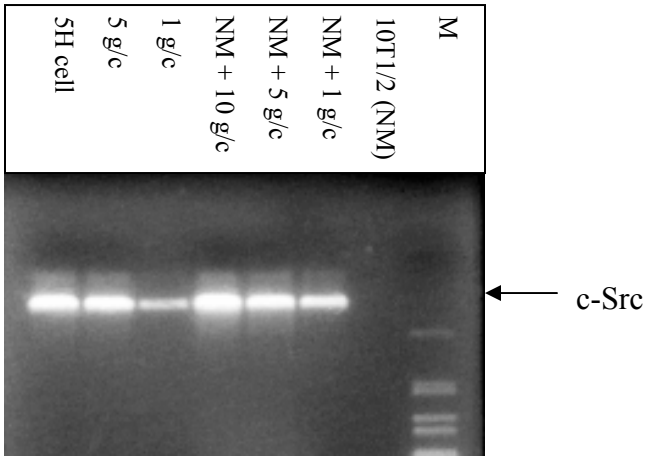


Figure D. PCR shows the presence of a chicken c-Src cDNA in genomic DNA derived from the 5H mouse fibroblast cell line, but not from the normal mouse (NM) 10T1/2 parental line.

I detected the presence of chicken c-Src only in the 5H cells, which shows the specificity of the reaction and as little as 1 gene copy. 1 gene copy is intended to create control template preparations, in which tail DNA is spiked with known amounts of transgene DNA to create single copy and multiple copy standards. These are used to verify that Southern blot or PCR reaction is sensitive enough to detect the integration of a single

copy of a transgene into mice genome. This will ensure that the screen for transgenic founders will not miss any transgenic lines of mice.

BSL1- STOPVENUS-c-Src transgenic mice will be gerated the same way as MMTV-c-Src transgenic mice. The Transgenic Mice Core Facility of the University of Virginia will assist me in all aspects of this phase of the project. I provided the facility with the expression vector, and they performed blastocyst injection and implantation of embryos into pseudopregnant females. I will test genomic DNA from tail crops by PCR and Southern blotting for the presence of c-Src transgene. Breeding of BSL1- STOPVENUS-c-Src mice with MMTV-Cre transgenic mice will result in MMTV-c-Src transgenic mice and targeted expression of c-Src (and VENUS) in mammary glands.

Task 3. To monitor tumor formation in MMTV-c-Src and MMTV-EGFR transgenic mice

a) None of the MMTV- EGFR transgenic mice developed tumors by the age of 2 years. They are being analyzed for the presence of displasia in mammary glands and other hormone responsible tissues
b) MMTV-c-Src will be monitored for the tumor formation after they will be generated.

Task 4. To generate EGFR/ c-Src bigenic mice and monitor tumor formation.

Because none of the MMTV c-Src transgenic mice, derived from MMTV-pMSG-Src plasmid, did not yield any positive pups, and new BSL1- STOPVENUS-c-Src mice are being generated, one of the alternative approaches was used to complete the project.

TGFα is a ligand for EGF receptor, and their synergistic effect was tested before in cell culture (published data), so it would be reasonable to expect increased tumor formation in bigenic MMTV-EGFR/TGFα transgenic mice compare to single transgenic mice.

I obtained MMTV-TGFα transgenic mice from Jackson Laboratories and I have established our lab's own colony of MMTV-TGFα transgenic mice.

I tested genomic DNA from tail crops by PCR for the presence of the TGFα transgene using human specific TGFa primers.

MMTV-human TGFα primers structure: TGF1 – AGTTCTGCTTCCATGCAACC; TGF2 – TGATGATAAGGACAGCCAGG

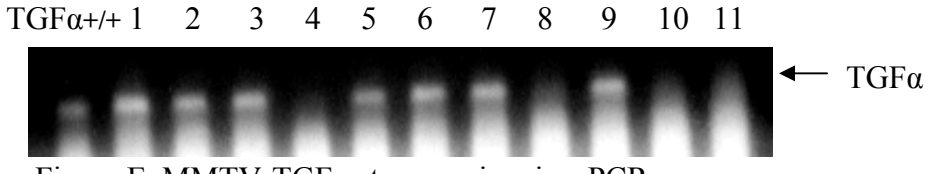


Figure E. MMTV-TGFα transgenic mice. PCR.

Human-specific primers for TGFα were used for PCR reactions to detect the presence of the transgene, against a mouse background. The MMTV- TGFα primers gave a PCR product of about 0.192 kb. 1-10 samples of transgenic mice genomic DNA, 11 –negative control, non-transgenic mice genomic DNA, TGFα+/+ – positive control for mice genomic DNA

24 of 38 (63%) pups from 4 mice-founders (2 Breeding couples+/+ +/-) were positive in initial screens. Positive pups where then interbred to obtain 100% transmission of the transgene. Embryos have been cryo-preserved.

Monitor tumor formation.
3 of 15 MMTV- TGFα transgenic mice developed tumors – histologically hemangiosarcoma; 2 died from pyogenic infections at the age of 6 month.

After establishing a colony of MMTV-TGFα transgenic mice, they were bred with MMTV-EGFR transgenic mice to derive bigenic MMTV-EGFR/ TGFα transgenic mice.

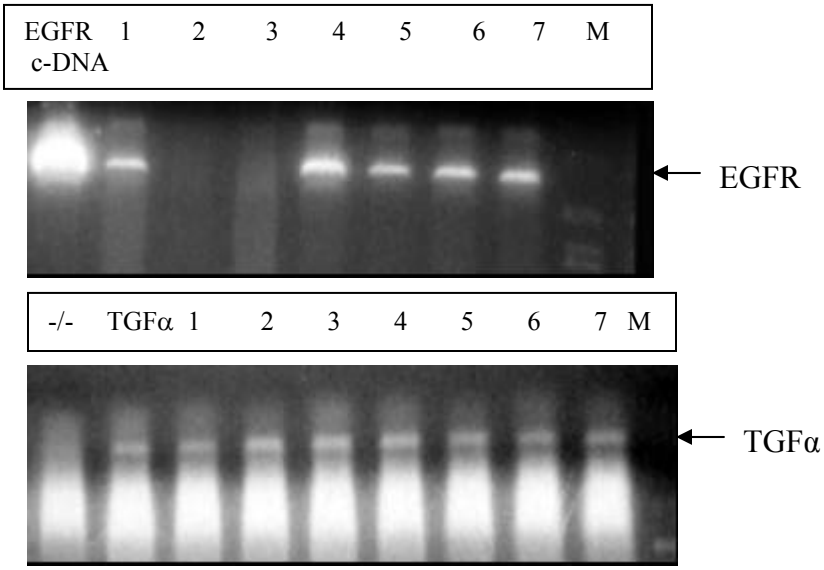


Figure F. MMTV-EGFR/TGFα bigenic transgenic mice. PCR.
1-7 samples of bigenic transgenic mice genomic DNA, EGFRcDNA- plasmid DNA, positive control; TGFα - transgenic mice genomic DNA, positive control; -/- nontransgenic mice genomic DNA, negative control M- marker

PCR reaction yields 5 of 7 positive mice for both EGFR and TGFa transgenes. These mice will be interbred to generate a line with 100% transmittance of the transgenes

Bigenic MMTV-EGFR/TGFα and MMTV-EGFR/TGFα will be examined for tumor formation in the mammary gland and other steroid hormone responsive tissues. If the synergism hypothesis is correct, bigenic mice should develop large tumors more rapidly than single transgenic mice and trigenic more rapidly then bigenic mice thus validating the synergism between c-Src, EGFR and TGFα as targets for future therapies.

Key research accomplishments.

1. BSL1- STOPVENUS-c-Src transgene has been constructed in plasmid vector and its expression was tested in cell culture.

2. PCR reactions were performed in order to detect the presence of c-Src cDNA against a background of total mouse genomic DNA.

3. MMTV-TGFα and MMTV-EGFR transgenic mice were generated. Presence of transgenes was confirmed by PCR and Southern Blot.

4. Bigenic MMTV-EGFR/ TGFα transgenic mice were generated. Presence of transgenes was confirmed by PCR.

5. The results of transgenic mice generation were presented at the scientific conference in Philadelphia, Pennsylvania "Era of Hope" – "Cooperative interaction of human epidermal growth factor receptor with Src tyrosine kinase and transforming growth factor alpha in breast tumorigenesis".

Reportable outcomes

1. List of reportable outcomes that have resulted from this award to include:

    a) Abstract from the conference in Philadelphia, Pennsylvania "Era of Hope" – "Cooperative interaction of human epidermal growth factor receptor with Src tyrosine kinase and transforming growth factor alpha in breast tumorigenesis".

Abstract is enclosed. See appendices.

Conclusions.

1. MMTV-TGFα and MMTV-EGFR transgenic mice were generated. Presence of transgenes was confirmed by PCR and Southern Blot.

2. Bigenic MMTV-EGFR/ TGFα transgenic mice were generated. Presence of transgenes was confirmed by PCR.

3. 3 from 15 MMTV-TGFα transgenic mice developed hemangiosarcomas, 2 died from pyogenic infections at the age of 6-8 month.

4. At the present time, none of the bigenic MMTV-EGFR/ TGFα transgenic mice have visible tumors at the age of 2 month; however, they will be monitored further for tumor formation up to the age of 2 years. They will be also examined for evidence of dysplasia, particularly in steroid-responsive tissues, such as mammary gland, uterus, ovary and prostate.

5. Interaction with other signaling molecules such as growth factors, intracellular transducers, or nuclear transcription factors may play a role in EGFR-induced tumorigenesis. To determine this, MMTV EGFR transgenic mice will be cross-bred with MMTV-c-Src, and MMTV-EGFR/ TGFα bigenic mice will be cross-bred with MMTV-c-Src transgenic mice to form bigenic or trigenic mice respectively, then examined for tumor formation in the mammary gland tissue. If the synergism hypothesis is correct, bigenic mice should develop large tumors more rapidly than single transgenic mice, and trigenic more rapidly then bigenic mice, thus validating the synergism between c-Src, EGFR and TGFα as targets for future therapies.

COOPERATIVE INTERACTION OF HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR WITH SRC TYROSINE KINASE AND TRANSFORMING GROWTH FACTOR ALPHA IN BREAST TUMORIGENESIS.

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The U.S. Army Medical Research and Materiel Command under DAMD 17-3-1-0253 supported this work